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Primary cultures of adult rat hepatocytes were used to investigate the comparative toxicity of three cyclic polypeptides (cyclosporine, gramicidin-s, microcystin-LR) and two depsipeptides (enniatin-b and valinomycin). Cell injury was assessed by the release of cellular [^{14}C] adenine nucleotides and lactate dehydrogenase into the media. At 1 μM, the cyclic polypeptides (cyclosporine and gramicidin-s) and depsipeptides (enniatin-b and valinomycin) did not induce a significant release of adenine nucleotides or lactate dehydrogenase from cultured rat hepatocytes as compared to controls. However, gramicidin-s, valinomycin, and cyclosporine induced significant cytotoxicity at 50 μM. Microcystin-LR dose-reponse studies indicated that maximum cytotoxicity was found at 1 μM. Comparatively, gramicidin-s, valinomycin and cyclosporine were at least 50 times less cytotoxic to rat hepatocytes than microcystin-LR. The release of [^{14}C]nucleotides from hepatocytes treated with microcystin-LR was distinctively different by the presence of a lag phase from that observed in hepatocytes treated with the other peptides.

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COMPARATIVE TOXICITY OF CYCLIC POLYPEPTIDES AND DEPSIPEPTIDES ON CULTURED RAT HEPATOCYTES

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Comparative Toxicity of Cyclic Polypeptides and Depsipeptides on Cultured Rat Hepatocytes. K.A. MEREISH, R. SOLOW, Y. SINGH AND R. BHATNAGER *Funda. Appl. Toxicol.* , , . Primary cultures of adult rat hepatocytes were used to investigate the comparative toxicity of three cyclic polypeptides (cyclosporine, gramicidin-s, microcystin-LR) and two depsipeptides (enniatin-b and valinomycin). Cell injury was assessed by the release of cellular [^{14}C] adenine nucleotides and lactate dehydrogenase into the media. At 1 μM, the cyclic polypeptides (cyclosporine and gramicidin-s) and depsipeptides (enniatin-b and valinomycin) did not induce a significant release of adenine nucleotides or lactate dehydrogenase from cultured rat hepatocytes as compared to controls. However, gramicidin-s, valinomycin, and cyclosporine induced significant cytotoxicity at 50 μM. Microcystin-LR dose-reponse studies indicated that maximum cytotoxicity was found at 1 μM. Comparatively, gramicidin-s, valinomycin and cyclosporine were at least 50 times less cytotoxic to rat hepatocytes than microcystin-LR. The release of [^{14}C]nucleotides from hepatocytes treated with microcystin-LR was distinctively different by the presence of a lag phase from that observed in hepatocytes treated with the other peptides.

Low-molecular-weight, cyclic polypeptides (peptides linked through an amide linkage) and depsipolypeptides (peptides linked through an ester linkage) comprise a small group of metabolites produced by fungi, algae, or bacteria. Among these cyclic polypeptides are cyclosporine, gramicidin-s, and microcystin-LR, while valinomycin and enniatin-b represent cyclic depsipeptides (Fig. 1). These cyclic compounds possess varied pharmacological properties ranging from antimicrobial activity (valinomycin, enniatin-b, gramicidin-s, microcystin-LR), strong immunosuppressive activity (cyclosporine), to antimalarial activity (valinomycin, cyclosporin, gramicidin-s). Many of these small mass (<1500 daltons) cyclic peptides possess ionophoric properties exhibiting differences in ion selectivity and affinities. The toxicity (LD50) of these compounds varies from microgram to milligram quantities in rodents. The LD50 for valinomycin (Daoud and Juliano, 1986), enniatin-b (Wannemacher *et al.*, 1988), cyclosporine (Ryffel, 1982), microcystin-LR (Runnegar and Falconer, 1981) in mice and the LD50 for gramicidin-s in rats (Merck Index, 1976, p. 4387), has been reported to be 1.7 mg/kg, 20.6 mg/kg ip, 107 mg/kg iv, 56 ug/kg ip, and 17 mg/kg ip respectively. Although mice treated with 200 mg/kg/day of cyclosporine (Boland *et al.*, 1984), or sublethal doses of microcystin-LR (Runnegar and Falconer, 1981), developed hepatic vascular congestion and fatty liver, there is no information available on the hepatotoxicity of the other cyclic polypeptides.

Mouse liver slices incubated *in vitro* with microcystin-LR exhibited no major defects in the synthesis of protein, RNA, DNA, or oxygen consupmtion (Runnegar and Falconer, 1981; 1982). Microcystin-LR induces in mice a rapid onset of liver damage (Falconer *et al.*, 1981; Runnegar and Falconer, 1981; Theiss 1984; Theiss and Carmichael, 1986) and necrosis of cultured hepatocytes after several hours of incubation with the toxin (Foxall and Sasner, 1981).

This study was designed to compare cell injury induced by these cyclic peptides and depsipeptides by using a simple and rapid assay to detect cell damage (Shirhatti and Krishna, 1985). The method is a noninvasive assay that evaluates injury to cultured cells grown as monolayers. The assay measures the release of [^{14}C]nucleotides from cells exposed to toxic agents. This *in-vitro* assay has been used to assess hepatocyte damage induced by exposure to toxic agents such as acetaminophen, the calcium ionophore A23187, or daunomycin (Shirhatti and Krishna, 1985). The release of nucleotides from cells exposed to various toxic agents has been shown to be an early marker occurring prior to or concomitant with cell damage (Chenery *et al.*, 1981). Although the release of other markers, such as radiolabeled proteins [^{125}I]iododeoxyuridine, or [^{3}H]thymidine labeled DNA, are frequently used to evaluate cytotoxicity (Schlager and Adams, 1983), these markers only begin to leak during or after nuclear disintegration and cell lysis. Therefore, the method described by Shirhatti and Krishna (1985) offers an advantage in investigating the comparative toxicity of toxic agents because it is able to detect cell damage prior to the onset of necrosis.

METHODS

Materials. The following materials were obtained commercially from the indicated sources: gramicidin-s (Chemical Dynamics Corp., South Field, NJ); valinomycin (Calbiochem, La Jolla, CA); [^{14}C]adenine (50 mCi/mmol, New England Nuclear, Boston, MA); tissue culture medium and fetal bovine serum albumin (GIBCO, Grand Island, NY); tissue culture ware (Becton-Dickinson Labware, Licoln Park, NJ) rat tail collagen, collagenase type IV , 5'-AMP, 5'-ADP, 5'-ATP, 5'-IMP, adenosine and adenine (Sigma, St. Louis, MO).

Male, FW.LEW, congenic, inbred (G. Anderson, USAMRIID, Fort Detrick, Frederick, MD) rats weighing between 250-300 g, were used for all

experiments. Microcystin-LR (85-95% purity by high-performance-liquid-chromatography) was obtained from Dr. W. Carmichael, Wright State University, Dayton, OH. The following materials were gifts from the indicatd sources: cyclosporine (Sandoz Laboratories, East Hanover, NJ) and enniatin-b (~95% purity by thin layer chromatography) from Dr. H.R. Burmeister, Northern Regional Research Center, USDA, Peoria, IL.

Hepatocytes. Rat hepatocytes were isolated and cultured according to the method of Elliget and Kolaja (1983). Viable hepatocytes were counted by trypan blue exclusion. The rinsed hepatocytes were resuspended at 5 x 10^5 viable cells per ml in L15 medium, containig 17% fetal calf serum, and seeded on collagen-coated, 6-well plates by adding 1 ml of cell suspension per well. The cells were allowed to settle for 30 min at room temperature and then incubated at 37°C with 5% CO_2 and 90% humidity for an additional 2 hr. After incubation, the majority of the cells had attached to the bottom of the well and formed a monolayer. The non-attached cells were removed by aspiration and 2 ml of fresh culture medium was added to each well.

Labeling the Nucleotide Pool and Measurement of Drug-Induced Toxicity. After overnight incubation of the hepatocytes, culture medium from each well was replaced with 1 ml of L15 medium containing [^{14}C]adenine (0.2 µCi, 4 µM). Adenine nucleotide pool was labeled as described by Shirhatti and Krishna (1985). Labeled cells were then incubated for 24 hr with 1 ml of medium containing various concentrations of cyclic peptides or with 1 ml medium as control. At selected time intervals, incubation was terminated and wells were assayed for toxicity indicies. Cell medium was removed and centrifuged at 500 x g for 4 min in an Eppendorf centrifuge, model 5414. An aliquot (200 µl) of the supernatant was removed and counted for radioactivity

in 10 ml of Hydrofluor (National Diagnostic, Somerville, NJ) in a Beckman scintillation counter, model LS5800 (Beckman Inst. Co, Fullertin, CA). Another aliquot (400 µl) of the supernatant was removed and stored at -4°C for nucleotides and lactate dehydrogenase (LDH) determination. The cells were lysed by the addition to each well of 1 ml of 0.05% digitonin in phosphate buffer. An aliquot of cell lysate was used to determine radioactivity, LDH, and protein content. Protein levels were determined by using Pierce protein reagent (Pierce, Rockford, IL) and bovine serum albumin as the standard. Lactate dehydrogenase was assayed with sodium lactate as substrate and NAD as the cofactor; the rate of formation of NADH was monitored at 340 nm with Cobas Bio (Roche Analytical Inst, Nutley, NJ).

[^{14}C]nucleotides (AMP, ADP, ATP, IMP), adenine and adenosine from hepatocytes' supernatant were determined by thin-layer chromatography on PEI-cellulose plates as described by Bochner and Ames (1982). Regions corresponding to the chromatographed standards were scraped from the plate and counted for radioactivity. Control and treated hepatocytes were examined under phase contrast microscopy (Nikon Diaphot inverted phase contrast microscope) to examine morphological changes.

RESULTS

Cultured rat hepatocytes incubated with 1 µM of valinomycin, gramicidin-s, enniatin-b, or cyclosporine over 24 hr incubation did not release [^{14}C]nucleotides (Fig. 2) or LDH (Fig. 3) greater than control levels or induce any morphological changes. Hepatocytes treated with 1 µM of microcystin-LR leaked a significant increase over time in the amount of [^{14}C]-nucleotide (Fig. 2) and LDH (Fig. 3) as compared to control cells. This increase was detectable 4 hr after exposure. After 10 hr of incubation, approximately 80% of the cellular [^{14}C]nucleotides was released from the

cells. A concentration of 0.1 μM of microcystin-LR also induced the release of both [^{14}C]nucleotides and LDH from cultured rat hepatocytes as compared to control cells (Fig. 4, 5). However, the release of [^{14}C]nucleotides and LDH was signifcant only after 10 and 8 hr, respectively (data not shown).

The dose-response relationship between microcystin-LR (0.1-50 μM) and the release of [^{14}C]nucleotides and LDH are summarized in Figures 4 and 5, respectively. Maximum dose-response for microcystin-LR was observed at 1 μM concentration for both markers.

In order to determine whether the other cyclic polypeptides and depsipeptides could induce on cultured rat hepatocytes, a similar toxic effect at higher dose levels, the cells were incubated with 50 μM of valinomycin, cyclosporine, or gramicidin-s for a total of 24 hr (enniatin-b was not tested at 50 μM, due to inadequate quantities). This concentration was chosen because it is close to the concentration required for pharmacological effects in biological systems. At 50 μM, valinomycin, cyclosporine, and gramicidin-s induced a significant release of [^{14}C]nucleotides from hepatocytes (Fig. 6) into the medium as compared to control cells. As shown in Figure 6, the effect was significant after 2 hr of incubation and maximal by 6 hr. Differences in the amounts and/or rates of [^{14}C]nucleotides released was observed among cells treated when each of the three cyclic polypeptides. Gramicidin-s- or valinomycin-treated cells released about 80% and 70% of total nucleotides, respectively, with the maximal rate of release observed within 2 hr of incubation. Cyclosporine induced a 90% release of [^{14}C]nucleotides from hepatocytes, with the maximal rate of release within 6 hr of incubation (Fig. 6).

At 50 μM, the three cylic polypeptides also induced a significant release of LDH from treated hepatocytes as compared to control cells (Fig. 7). Gramicidin-s- treated cells released LDH at a rate and in amounts which

paralleled to the release of [^{14}C]nucleotides. There was a significant lag in the release of LDH compared to the release of nucleotides from cells treated with valinomycin or with cyclosporine. Furthermore, in cells treated with cyclosporine and valinomycin, the reduced amounts of LDH released at early time points (2 and 4 hr) were significantly less than the released amounts of nucleotides. Significant LDH release from valinomycin-, microcystin-LR-, and cyclosporine-treated cells was detected at 4 hr, with the maximal rate of release observed between 4 and 8 hr of incubation. However, the maximal rate of LDH release in gramicidin-s-treated cells was within 2 hr of incubation.

The Rf values for AMP, ADP, ATP, IMP, and adenosine were 0.68, 0.34, 0.1, 0.58, and 0.54, respectively. Due to the poor resolution in separating adenosine from IMP, the bands corresponding to these two compounds was counted as one and reported as IMP. The majority of [^{14}C]nucleotides released into the medium from control or treated cells was determinated as AMP, IMP and/or adenosine. The distribution of labeled nucleotides released into the medium was the same in control and treated cells (AMP and IMP, 89%; ADP, 8%; ATP, 0.5%).

DISCUSSION

Microcystin-LR has been shown to be hepatotoxic selectively (Theiss and Carmichael, 1986). At 1 μM, microcystin-LR-treated hepatocytes released 95% of both [^{14}C]nucleotides and LDH. The same concentration of gramicidin-s, cyclosporine, valinomycin, and enniatin-b did not affect the release of either markers. The effect of microcystin-LR on hepatocytes may have been due to (a) the presence of specific receptors for microcystin-LR; (b) significant bioactivation of microcystin-LR into toxic metabolite(s), with or without

concomitant depletion of critical cofactors required for cell viability, i.e., glutathione, and/or NADPH or (c) microcystin-LR induced synthesis and/or the release of abnormal levels of biologically toxic mediator(s), i.e., prostaglandines, leukotrienes, radicals, etc.

Hepatocytes treated with 1 μM microcystin-LR exhibited a parallel pattern of release of [^{14}C]nucleotides and LDH (Fig. 2, 3). Both the percent and the kinetics of release from treated cells were similar for both markers. This parallel release of markers suggests that at this concentration (1 μM) microcystin-LR damaged hepatocytes, at least in part by damaging the plasma membrane. Hepatocytes incubated with 1 μM microcystin-LR for 2 hr released a significant amount of arachidonic acid, and enhanced the formation of prostacyclin, prostaglandin E2, and thromboxane B2 as compared to control cells (Naseem *et al.*, 1988). Hepatocytes treated with 0.1 μM microcystin-LR did not release [^{14}C]nucleotides or LDH until 8 hr of toxin exposure (data not shown).

The presence of a lag phase before the release of markers from cells treated with low toxin concentrations may indicate that the mechanism of hepatotoxicity is a mediated mode of action. Interestingly, although a significant amount of arachidonic acid and thrombaxane B2 were released from hepatocytes incubated with 0.1 μM microcystin-LR for 2 hr, at this concentration, the toxin did not induce significant release of other prostaglandins (Naseem *et al.*, 1988), [^{14}C]nucleotides, or LDH.

A dose-response, toxicity-relationship study in isolated hepatocytes indicated that microcystin-LR induced maximum release of toxicity markers at ≤1 μM concentration. Comparatively, at least 50X microcystin-LR dose of valinomycin, cyclosporine, or gramicidin-a was necessary to achieve the same cytotoxicity induced by microcystin-LR in cultured rat hepatocytes.

Hepatocytes treated with 50 μM of gramicidin-s, valinomycin, or cyclosporine released significant amounts of both [^{14}C]nucleotides and LDH, indicating the hepatotoxicity of these cyclic polypeptides and depsipeptides. Gramicidin-s (50 μM) treated hepatocytes released 95% of both [^{14}C]nucleotides and LDH in a parallel fashion within the first 2-4 hr of incubation. In addition to the absence of a lag time in the release, the parallel release of both markers suggests that gramicidin-s may induce cell damage via a detergent effect on the plasma membrane. This is supported by several studies of the effect of micromolar amounts of gramicidin-s on intact cells and model membranes. At 50 μM, gramicidin-s was shown to induce 80% release of 5,6-carboxyfluoroscein from phosphatidyl choline large, unilamellar vesicles (Eytan and Broza, 1988). Additional evidence for a putative detergent effect of gramicidin-s comes from studies investigating the effect of gramicidin-D on intact red blood cells and red blood cell membranes (Classen *et al.*, 1987). Thus, exposure of hepatocytes to 50 μM of gramicidin-s could possibly lead to the formation of gramicidin-s aggregates in the plasma membrane, which could induce a large leakage and permit the simultaneouse release of both [^{14}C]nucleotides and LDH.

Like that observed with gramicidin-s, the release of [^{14}C]nucleotides from hepatocytes incubated with 50 μM valinomycin or 50 μM cyclosporine occurred without a lag time. In contrast to nucleotide release, LDH release from valinomycin- and cyclosporine- treated hepatocytes occurred after a lag phase. The fact that [^{14}C]nucleotides release preceded the release of LDH suggests that the disruption induced by valinomycin and cyclosporine of hepatocytes plasma membrane required time before the perturbation was sufficient to allow the leakage of LDH.

In intact cells and membrane-bound enzymes, valinomycin has been shown to have a variety of effects that are not related to its ionophoric property.

Valinomycin at 10^{-7} M has been shown to reduce total cellular ATPase content by about 30% in a variety of cells (Kleuser *et al.*, 1985), reduced total cellular ATP levels in lymphocytes and depolarized mitochondria, and inhibited Con A mitogenic stimulation of lymphocytes without inducing cell damage (Felber *et al.*, 1982; Negendank *et al.*, 1982). Valinomycin at 10 μM promoted the incorporation of exogenous fatty acids into red blood cells phospholipid and increased fatty acid turnover in membranes, while at 3 μM, it inhibited the Ca^{2+}-ATPase of endoplasmic reticulum membranes by binding to the enzyme and modifying its affinity and specificity to monovalent cationic sites (Davidson and Berman, 1985). It is possible that 50 μM valinomycin induced hepatocyte damage by perturbing plasma membrane phospholipid turnover, binding and inhibiting critical membrane enzymes and/or depleting cellular ATP, as indicated by the release of [^{14}C]nucleotides into the medium.

Cyclosporine has been shown to induce reversible liver and kidney damage in 20 to 40% of patients receiving chronic therapeutic doses and in animals (Calne *et al.*, 1979; Thomson *et al.*, 1984). Cyclosporine also damages the liver, as shown by increased alkaline phosphatase and serum bilirubin levels and dilation of endoplasmic reticulum (Blair *et al.*, 1982). At subtoxic doses, hepatocytes have been shown to take up cyclosporine in a temperature-dependent diffusion process, where 50% of cyclosporine was found to partition within the cytoplasmic-soluble fraction and 50% within the plasma membrane (Ziegler *et al.*, 1988). Also, Nagelkerke *et al.*, (1987) demonstrated that cyclosporine binds extensively to lipoproteins and other macromolecules. Furthermore, depletion of glutathione increased the coavalent binding of cyclosporine metabolites to hepatocyte macromolecules (Nagelkerke *et al.*, 1987). Since cyclosporine has been shown to partition in membrane lipid, it is possible that it mediates its toxicity by disrupting the membrane

permeability barrier. The release of LDH may proceed some of these events which are associated with [^{14}C]nucleotides release.

The release of [^{14}C]nucleotides from hepatocytes treated with 50 μM microcystin-LR was different from that induced by gramicidin-s, valinomycin, or cyclosporine by the presence of a prominent lag time. The lag phase was also observed with hepatocytes incubated with 0.1 - 50 μM microcystin-LR. This lag phase suggests that cell damage induced by microcystin-LR might be via a different mechanism than damage caused by the other cyclic peptides. The expression of hepatotoxicity by carbon tetrachloride, bromobenzene, and acetaminophen is dependent upon extensive metabolic activation, is concentration dependent, and exhibits a lag time for all these xenobiotics (Casini *et al.*, 1982; Moore *et al.*, 1985; Cherry *et al.*, 1981). It is possible that the *in-vivo* release of nucleotides from hepatocytes has biological significance, since, adenine nucleotides not only influence platelet aggregation, but are also potent vasoactive agents. Adenine nucleotides released from endothelial cells have been shown to act as local hormones which increase blood flow in muscle cells during hypoxia (Clemens and Forrester, 1979). The hemorrhagic liver produced in animals administered with microcystin-LR could be the result of a cascade of events initiated in hepatocytes. One of these events, is the efflux of adenine nucleotides, prostaglandin synthesis, and, finally, increased blood flow in liver. Exposure of cells labeled with [^{14}C]nucleotides to microcystin-LR, valinomycin, cyclosporine, enniatin-b or gramicidin-s did not seem to alter the percent distribution of released nucleotides. The majority of the released [^{14}C]nucleotides was associated with the AMP and IMP fractions. This observation is in agreement with that offered for hepatocytes exposed to other hepatotoxins such as daunomycin, acetaminophen, and A23187 (Shirhatti and Krishna, 1985).

This study indicated that the cyclic polypeptides and depsipeptides induced hepatocyte damage, mainly through membrane perturbation. Microcystin-LR causes hepatocyte damage by some mechanism that requires a lag time for toxin to be distributed into the cell, and biotranformed, and/or the release of a mediator(s).

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FIG. 1. Structural formulas of cyclic polypeptides and depsipeptides.

FIG. 2. Effect of 1 μM of microcystin-LR (★--★), cyclosporine (●---●), gramicidin-s (O—-O), valinomycin (■--■), or enniatin-b (▲...▲) on the release of [^{14}C]nucleotides from cultured rat hepatocytes. The release of [^{14}C]nucleotides from control (▼——▼) hepatocytes was monitored. Cells were incubated with [^{14}C]adenine for 1 hr. The hepatocytes were then washed and reincubated in 1 ml medium containing the selected polypeptides. At various times cell supernatants were collected, centrifuged, and the amount of [^{14}C]nucleotides released was determined as described in the text. Cells were lysed with digitonin and cellular [^{14}C]nucleotides was determined. The results are presented as the percent of [^{14}C]nucleotides released. Each point represents the mean of three determinations ± SD.

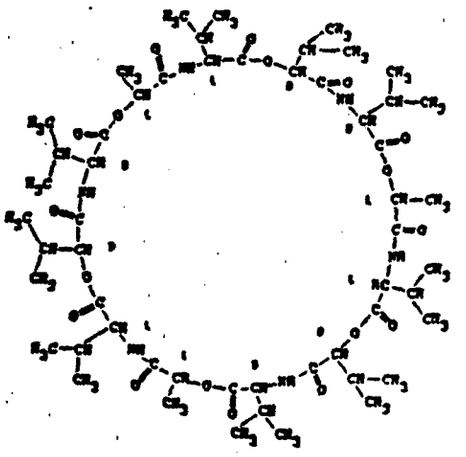
FIG. 3. Effect of 1 μM microcystin-LR (★--★), cyclosporine (●—●), gramicidin-s (O—-O), valinomycin (■--■), enniatin-b (▲...▲) or untreated (▼——▼) on the release of LDH from cultured rat hepatocytes. Cells were treated as decribed in Fig. 1. Each point represents the mean of three determinations + SD.

FIG. 4. Effect of various concentrations of microcystin-LR (0.1 - 50 μM) on the release of [^{14}C]nucleotides from cultured rat hepatocytes. Cells were treated as described in Fig. 1. Each point represents the mean of three determinations + SD.

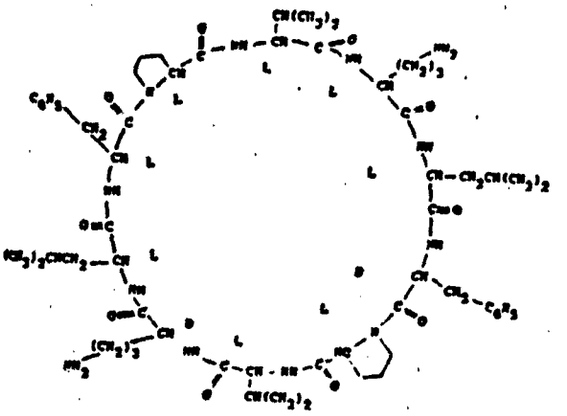
FIG. 5. Effect of various concentrations of microcystin-LR (0.1 - 50 μM) on the release of LDH from cultured rat hepatocytes. Cells were treated as described in Fig. 1. The results represent the mean of three determination + SD.

FIG. 6. Effect of 50 μM microcystin-LR (★–★), cyclosporine (●–●), gramidicin-s (○–○), valinomycin (■–■) or untreated (▼–▼) on the release of [^{14}C]nucleotides from cultured rat hepatocytes. Cells were treated as described in Fig. 1. Each point represents the mean of three determinations + SD.

FIG. 7. Effect of 50 μM microcystin-LR (★–★), cyclosporine (●–●), gramidicin-s (○–○), valinomycin (■–■) or untreated (▼–▼) on the release of LDH from cultured rat hepatocytes. Cells were treated as described in Fig. 1. Each point represents the mean of three determinations + SD.



Valinomycin



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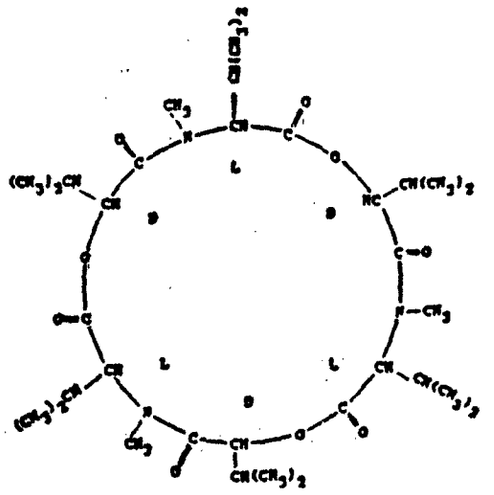

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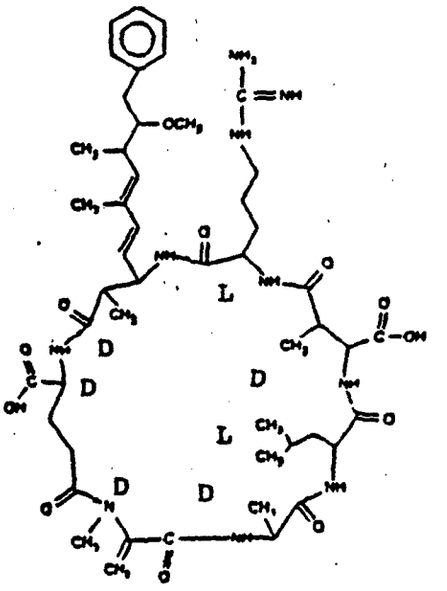

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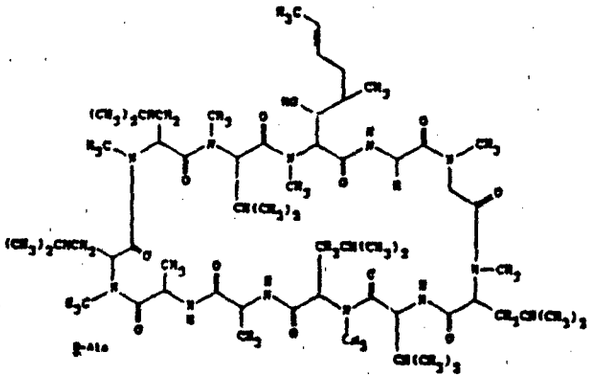
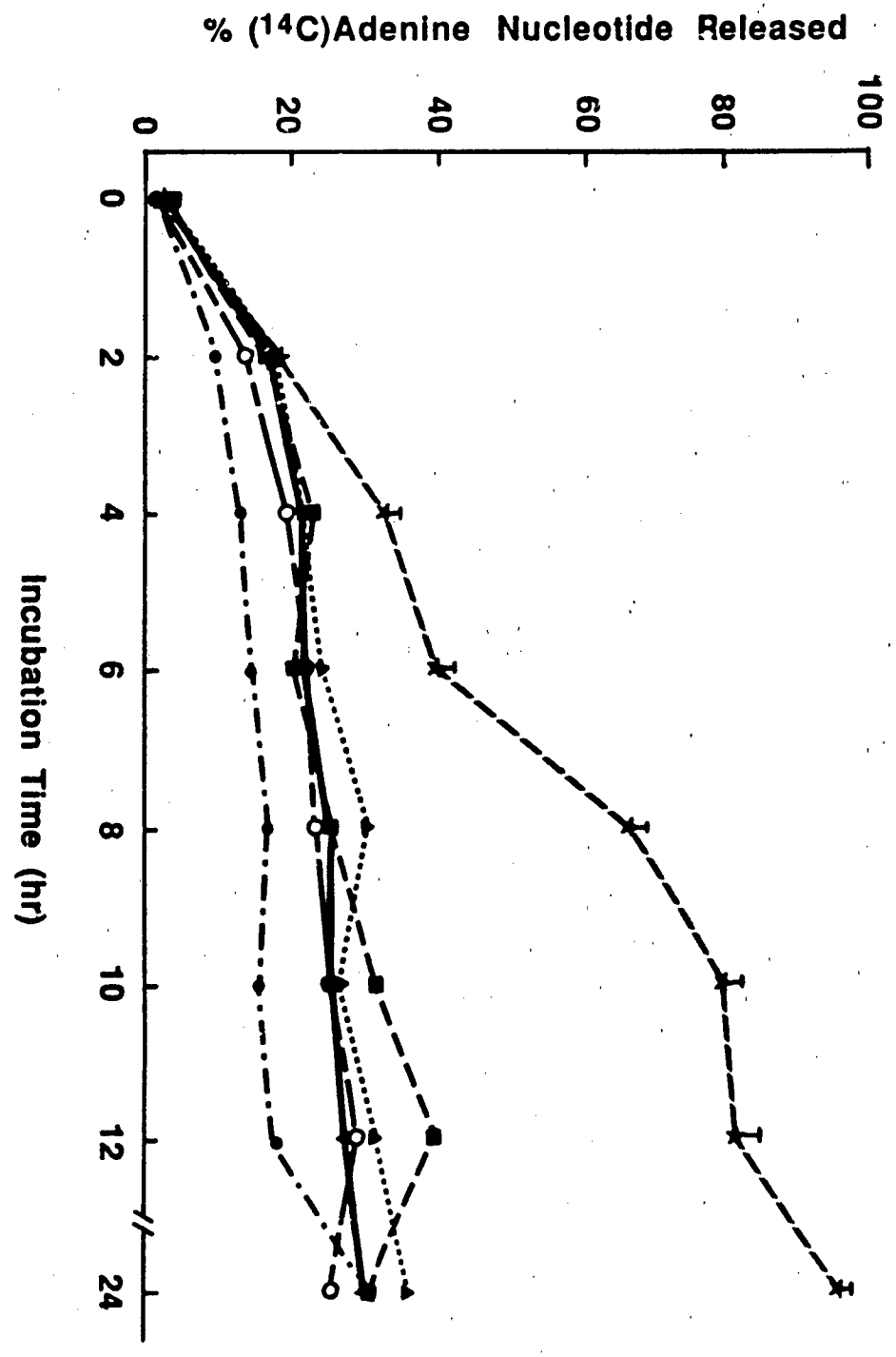
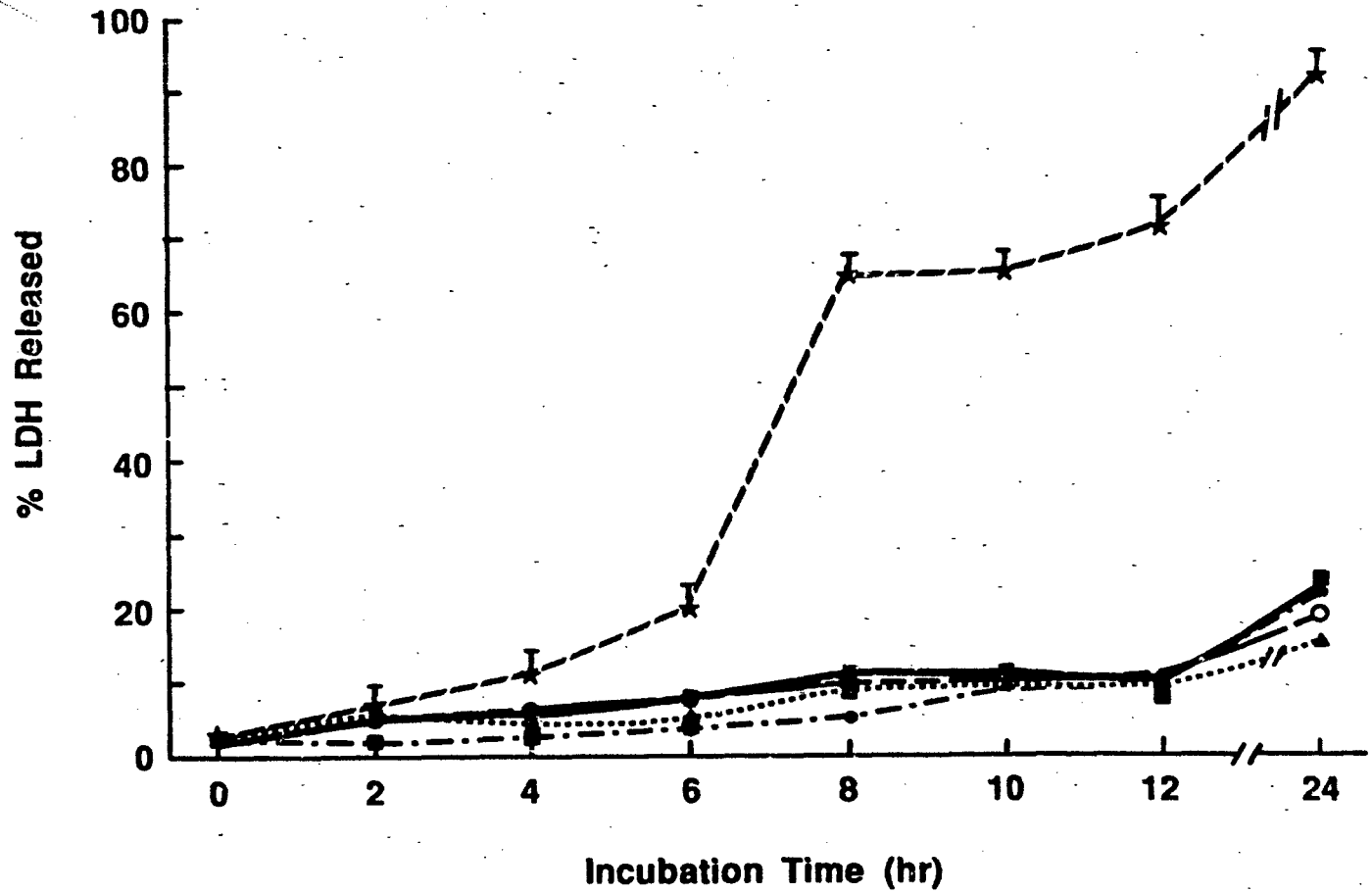
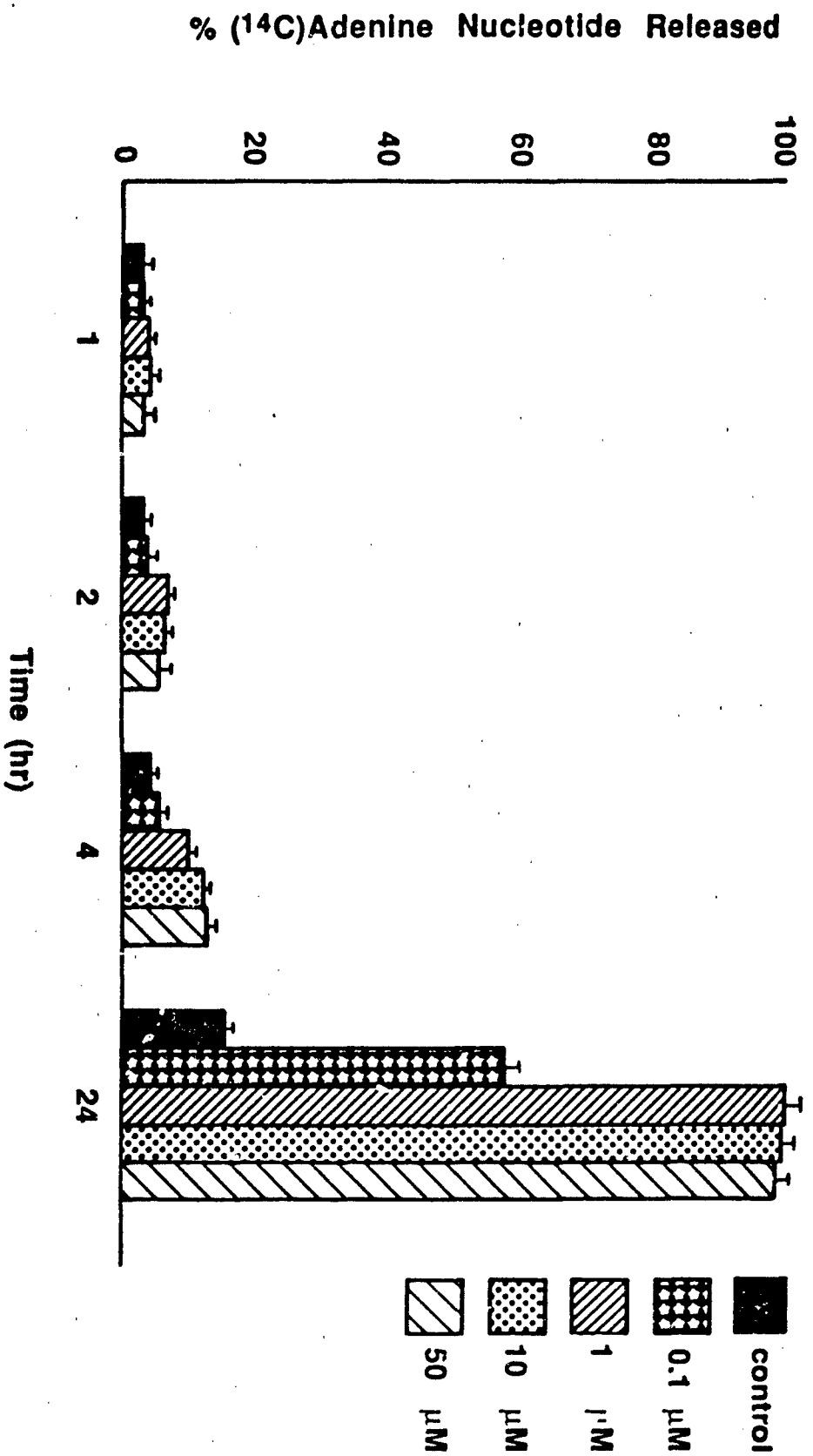
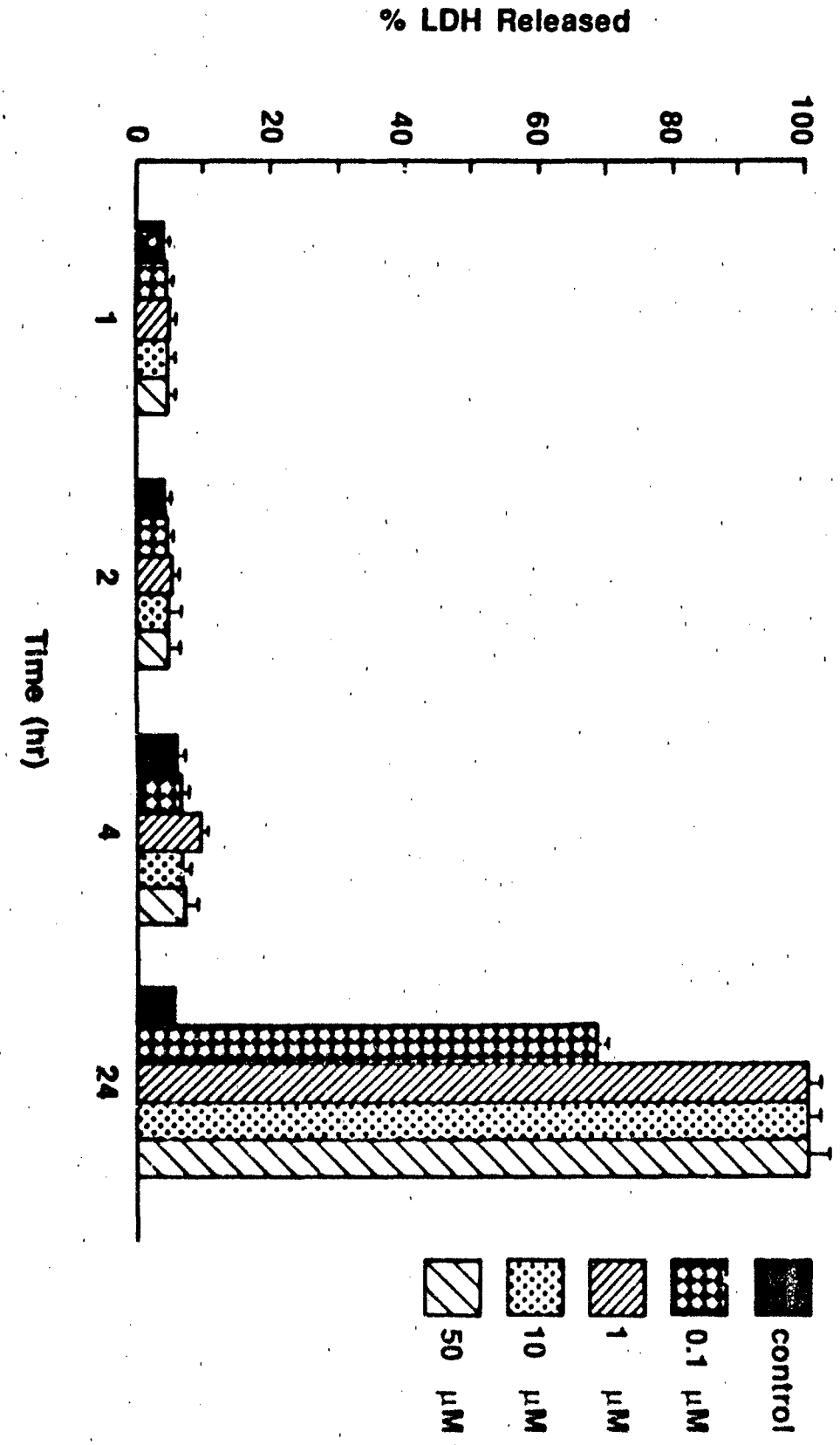


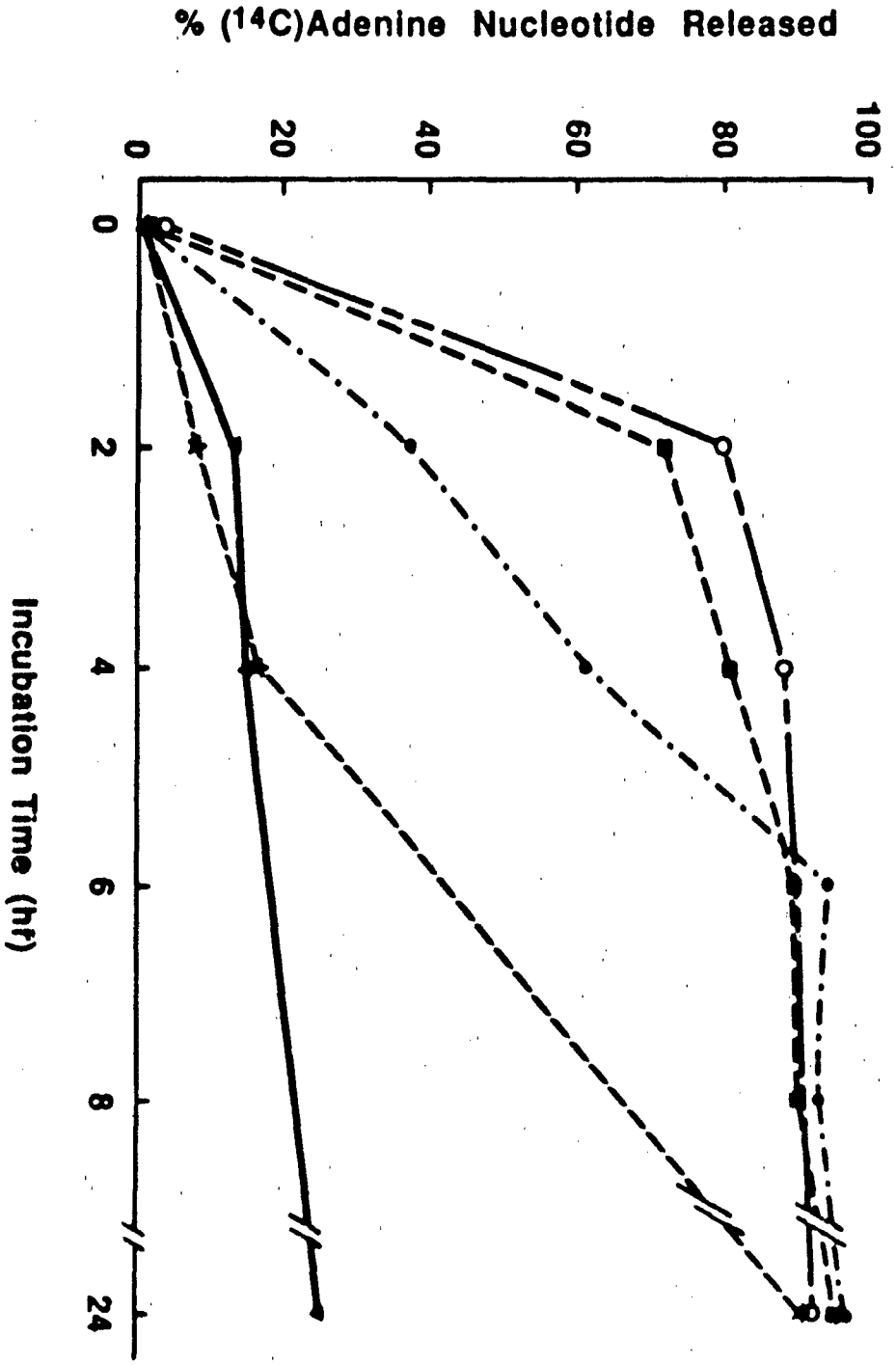
Cyclosporine

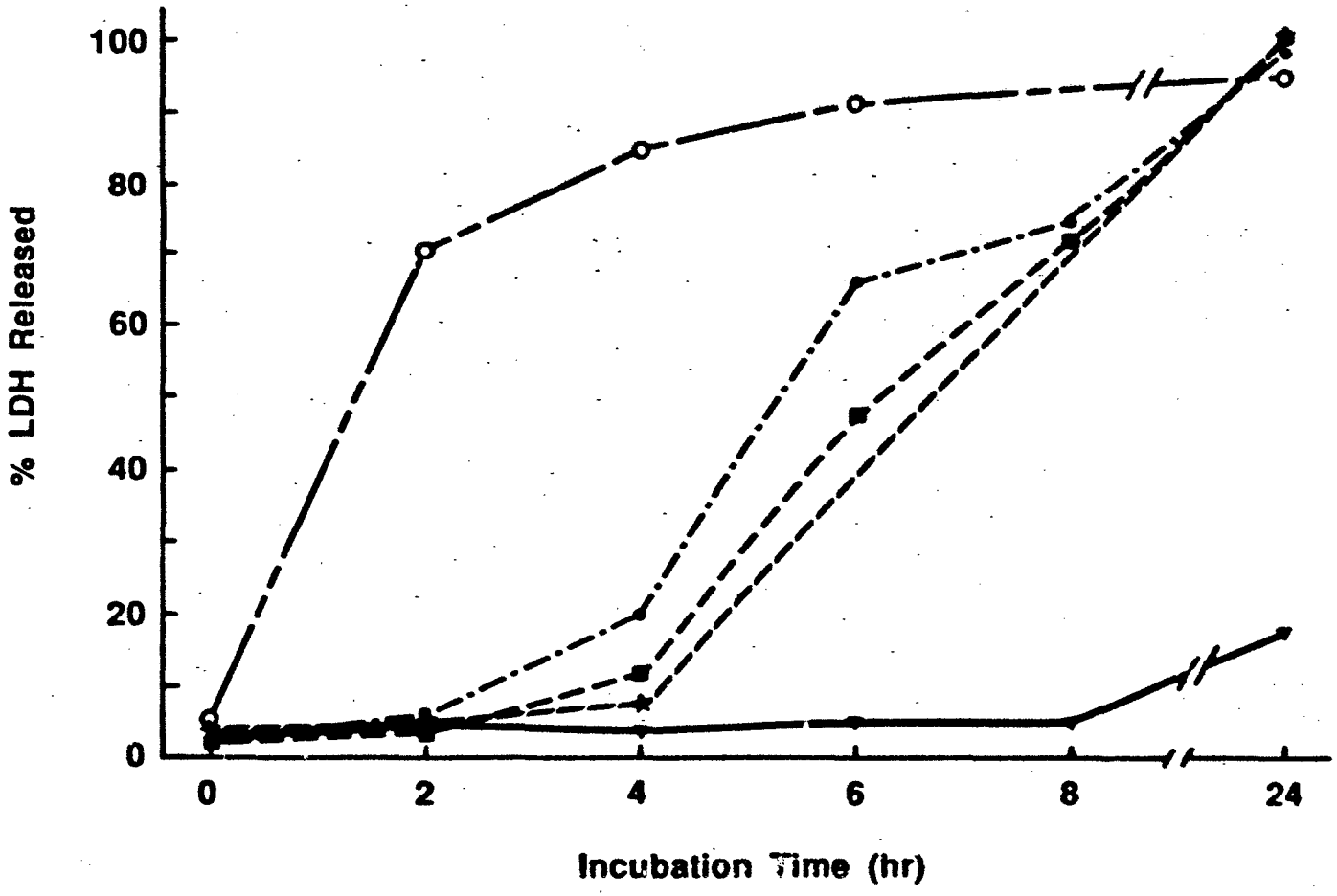

% (14C)Adenine Nucleotide Released
0
20
40
60
80
100
Incubation Time (hr)
0
2
4
6
8
10
12
24


100
80
60
40
20
0
% LDH Released
0
2
4
6
8
10
12
24
Incubation Time (hr)


% (14C)Adenine Nucleotide Released
0
20
40
60
80
100
Time (hr)
1
2
4
24
control
0.1 µM
1 µM
10 µM
50 µM


% LDH Released
0
20
40
60
80
100
Time (hr)
1
2
4
24
control
0.1 µM
1 µM
10 µM
50 µM


% (14C)Adenine Nucleotide Released
0
20
40
60
80
100
0
2
4
6
8
24
Incubation Time (hr)


100
80
60
40
20
0
% LDH Released
0
2
4
6
8
24
Incubation Time (hr)